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The Clouston syndrome mutation connexin30 A88V leads to hyperproliferation of sebaceous glands and hearing impairments in mice

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ABSTRACT

Distinct mutations in the gap junction protein connexin30 (Cx30) can cause the ectodermal dysplasia Clouston syndrome in humans. We have generated a new mouse line expressing the Clouston syndrome mutation Cx30A88V under the control of the endogenous Cx30 promoter. Our results show that the mutated Cx30A88V protein is incorporated in gap junctional plaques of the epidermis. Homozygous Cx30A88V mice reveal hyperproliferative and enlarged sebaceous glands as well as a mild palmoplantar hyperkeratosis. Additionally, homozygous mutant mice show an altered hearing profile compared to control mice. We conclude that the Cx30A88V mutation triggers hyperproliferation in the skin and changes the cochlear homeostasis in mice.

1. Introduction

Clouston's hidrotic ectodermal dysplasia (HED, also called Clouston syndrome, OMIM #129500) is a rare autosomal dominant genetic disorder characterized by alopecia, nail dystrophies and palmoplantar hyperkeratosis. Some patients develop hyperpigmentation of the skin over the large joints [1]. Additionally, sensorineural hearing loss has been reported in a few cases [2,3]. The extent of the symptoms can be highly variable even within the same family, i.e. the hair loss can be partial or total and can be manifested at birth or later [4]. Molecular genetic screening of Clouston syndrome patients revealed that the patients carried an autosomal dominant mutation in the GJB6 gene, which encodes the gap junction protein connexin30 (Cx30) [5–7].

Gap junctions are transmembrane channel proteins, which mediate cell–cell communication. They form conduits in the plasma membrane of two adjacent cells allowing the diffusional exchange of molecules up to a mass of 1.8 kDa [8]. Gap junctions are composed of connexin protein subunits. Six connexins build a hemichannel (connexon), which can interact with a hemichannel of a contacting cell to form a complete intercellular gap junction channel [9]. Connexins are members of a multigene family. So far, 20 different connexin isoforms have been described in mice and 21 have been found to be expressed in humans [10]. Each connexin isoform shows a cell type specific expression and distinct contribution to channel properties [11].

So far, four different Clouston syndrome-causing human Cx30 mutations have been identified: Cx30G11R, Cx30A88V, Cx30V37E and Cx30D50N [5–7]. Moreover, another Cx30 mutation (Cx30T5M) has been described to lead to hearing impairments in humans. However, in contrast to the Clouston syndrome associated mutations, patients harboring the Cx30T5M mutation exhibit no hair and skin symptoms [12]. Mutations in connexins can alter the channel properties of gap junctions. Coupling analyses of the mutations Cx30G11R and Cx30A88V in HeLa cells and in paired *Xenopus* oocytes revealed that these mutations do not influence the formation of functional intercellular channels, but cause open hemichannels as indicated by a leakage of ATP into the extracellular medium [13].

In order to analyse the effects of Cx30A88V *in vivo*, we inserted this mutation into the mouse genome. After ubiquitous

Abbreviations: Cx, Connexin; Cx30A88V, substitution of the amino acid residue alanine to valine at position 88 of Cx30; SG, sebaceous gland; ABR, Auditory Brainstem Responses; DPOAE, Distortion Product Otoacoustic Emissions; NLS, Nuclear Localization Signal; IRES, Internal Ribosomal Entry Site

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Cre-mediated deletion of the Cx30 wildtype coding region, the Cx30A88V mutation is expressed under control of the endogenous Cx30 promoter. Homozygous Cx30A88V mice exhibit several symptoms of the highly variable Clouston syndrome, for example a mild hyperkeratosis of the palmoplantar skin and hearing impairment, whereas other characteristics like hair loss and nail defects are not displayed by this mouse line.

2. Material and methods

The material and methods are summarized. Detailed descriptions of all methods can be found in the Supplemental material.

2.1. Treatment of mice

All experiments were performed with littermates of >87.5% CD-1 background.

All mice used in this study were kept under standard housing conditions with a 12 h/12 h dark-light cycle and with food water *ad libitum*. All experiments were carried out in agreement with local and state regulations for research with animals.

2.2. Histological and immunofluorescence analyses

For histological and immunofluorescence analyses skin biopsies were taken from the dorsal mid-thoracic region and from the paws.

For histological analyses the skin biopsies were fixed in Bouin's solution and embedded in paraffin or in 2-Hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). The 5 μm thick sections were stained with haematoxylin/eosin (HE).

For immunofluorescence analyses skin biopsies were frozen in Tissue-Tek embedding medium (Sakura, Zoeterwonde, The Netherlands), fixed in 4% paraformaldehyde and cryosectioned (14 μm). The following primary antibodies were used: mouse anti-Cx26 (monoclonal, 1:500, Invitrogen, Carlsbad, CA, USA, Cat. No. 13-8100), rabbit anti-Cx30 (diluted 1:250, Invitrogen, Cat. No. 71-2200), rabbit anti-keratin1 (diluted 1:500, Covance CRP, Muenster, Germany, Cat. No. PRB-149P), guinea pig anti-keratin K2e (diluted 1:200, Progen, Heidelberg, Germany, Cat. No. GP-CK2e), mouse anti-keratin 6 (monoclonal, diluted 1:100, Progen, Cat. Co. 61090), rat anti-Ki67 (monoclonal, diluted 1:50, DakoCytomation, Glostrup Denmark, Cat. No. M7249), rabbit anti-ß-galactosidase (diluted 1:500, MP Biomedicals, Santa Ana, CA, USA, Cat. No. 55976). The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG (diluted 1:1000, Invitrogen, Cat. No. A-11029, USA), Alexa Fluor 594 goat anti-rabbit IgG (diluted 1:1000, Invitrogen, Cat. No. A-11037, CA, USA), Alexa Fluor 488 goat anti-rat IgG (diluted 1:1000, Invitrogen, Cat. No. A-11006, USA), Alexa Fluor 488 goat anti-rabbit IgG (diluted 1:1000, Invitrogen, Cat. No. A-11008,), Cy2 goat anti-guinea pig IgG (diluted 1:1000, Jackson ImmunoResearch, West Grove, PA, USA).

For Nile Red stainings, cryosections were fixed in 4% paraformaldehyde, incubated for 2 h with 5 μg/ml Nile Red solution (Sigma–Aldrich, Cat. No. 72485, St. Louis, MO, USA) and mounted with Glycergel mounting medium (DakoCytomation, Glostrup, Denmark).

2.3. Immunoblot analyses

Total protein were extracted from paw skin. Extraction, electrophoresis, blotting and detection is described in the supplement. The following primary antibodies were used: mouse anti-Cx26 (monoclonal, 1:500, Invitrogen, Cat. No. 13-8100), rabbit anti-keratin1 (diluted 1:500, Covance CRP, Cat. No. PRB-149P), guinea pig anti-keratin K2e (diluted 1:1000, Progen, Cat. No. GP-CK2e) and mouse anti-keratin 6 (monoclonal, diluted 1:100, Progen, Cat. Co. 61090). Loading controls were performed with mouse anti-GAPDH (diluted 1:10,000, Millipore, Temecula, CA, USA) antibodies.

Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit, goat anti-giunea pig, diluted 1:10000, Jackson ImmunoResearch, West Grove, PA, USA) were used.

2.4. Hearing analyses

ABR (Auditory Brainstem Responses) were recorded as described in [14]. In brief, mice aged 6–9 weeks were anesthetized with Ketamine/Xylazine i.p. and the difference potential between subcutaneous electrodes at the mastoid and vertex was recorded during free-field stimulation. ABR threshold was determined with 10 dB precision as the lowest stimulus intensity that evoked a reproducible response waveform. For DPOAE we used the System III and the MF1 speaker system (Tucker Davis Technologies) driven by custom-written Matlab software (Mathworks) to deliver two continuous primary tones (frequency $f2 = 1.2 \times f1$, intensity $l2 = l1$-10 dB SPL) and sampled the signal using a MKE-2 microphone (Sennheiser) and sound card (DMX 6Fire, Terratec).

2.5. Statistical analyses

For statistical analyses a two-tailed Student's *t*-test or individual Mann Whitney *U* tests were used. A P-value < 0.05 was regarded as statistically significant. Asterisks indicate a P-value of <0.05 (*), <0.01 (**) or <0.001 (***). Error bars indicate standard error of the mean (S.E.M.).

3. Results

3.1. Generation of a Cx30A88V mouse line

Cx30A88V mice were generated by targeting the Cx30 locus in mouse embryonic stem (ES) cells via homologous recombination (Supplemental Fig. 1A). The conditional targeting vector contains the wildtype Cx30 sequence flanked by loxP sites and the coding region of the mutated Cx30A88V. The point mutation in the Cx30A88V gene, leading to a substitution of an alanine residue on position 88 by a valine residue, was inserted together with a ScaI restriction site by mutagenesis PCR. Therefore the ScaI restriction site is not present in the wildtype sequence of Cx30. The expression of Cx30A88V is linked to the expression of a lacZ reporter gene by an internal ribosomal entry site (IRES) between the coding region of Cx30A88V and a lacZ gene. The lacZ gene is coding for ß-galactosidase which, due to a nuclear localization signal (NLS), is transported to the nucleus and can be used to monitor the Cx30A88V expression in vivo. For selection of homologously recombined ES-cells, the vector includes cDNA coding for neomycin resistance, which is flanked by frt-sites.

HM1 ES-cells were transfected with the conditional Cx30A88V vector. Five out of 800 clones were positive (verified by PCR and Southern blot analyses, data not shown) and could be used for blastocyst injections. The heterozygous Cx30 +/floxA88V chimeras were mated with Flp-recombinase expressing mice to delete the neomycin resistance cDNA (Supplemental Fig. 1B) and with phosphoglycerate kinase (pgk)- cre recombinase expressing mice for ubiquitous deletion of the wildtype Cx30 sequence (Supplemental Fig. 1C). Cx30A88V mice were genotyped by PCR analyses (Supplemental Fig. 1D). To verify the presence of the mutated Cx30A88V sequence a PCR reaction amplifying the coding region of Cx30wt and Cx30A88V was performed, followed by a ScaI restriction (Supplemental Fig. 1E). The different genotypes of transgenic Cx30A88V

mice were further verified by Southern blot analyses (Supplemental Fig. 1F).

The heterozygous Cx30+/A88V mice were backcrossed to a CD-1 background. All mice used for analyses (wildtype, heterozygous or homozygous Cx30A88V mice) had a CD-1 background of >87.5%. Breeding of heterozygous Cx30+/A88V mice resulted in homozygous Cx30A88V/A88V mice which were viable, fertile and born at the expected Mendelian ratio.

3.2. Cx30A88V is located in gap junction plaques of the plasma membrane

In order to investigate the localization of the Cx30A88V protein, immunofluorescence stainings on paw sections using antibodies against Cx26 (Fig. 1A and B) and Cx30 (Fig. 1C and D) were performed. In the thick palmoplantar epidermis Cx26 and Cx30 co-localize in the membranes of suprabasal keratinocytes (Fig. 1E). In homozygous Cx30A88V mice, where only the mutated Cx30A88V is expressed, Cx30A88V co-localizes with Cx26 in the plasma membrane (Fig. 1F). No difference in the expression pattern of Cx30 or Cx30A88V and Cx26 could be observed between wildtype and homozygous Cx30A88V mice.

Since a recent publication proposes an inhibiting effect of genetic modifications of the Cx30 gene on the Cx26 gene expression [15], we analysed a putative impact of the Cx30A88V construct on the Cx26 gene. Immunoblot analyses of Cx26 of the liver of Cx30A88V mice were performed showing no alterations of the Cx26 expression in the liver of transgenic Cx30A88V mice compared to Cx30 wildtype littermates (Supplemental Fig. 2). Therefore, we conclude that the Cx30A88V construct does not affect the expression of the Cx26 gene locus.

3.3. Hyperproliferation of hairfollicle associated sebaceous glands

Homozygous Cx30A88V mice exhibited greasy fur compared to wildtype littermates (Fig. 2A). This phenotype could be already

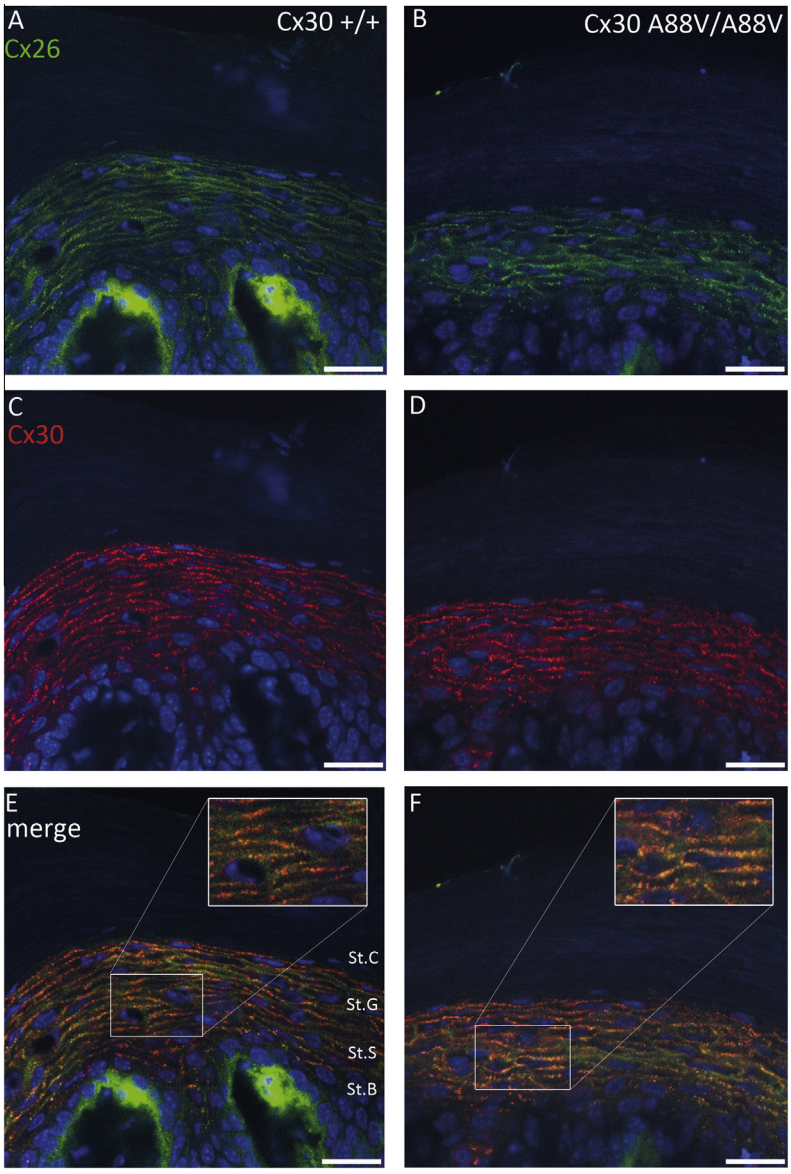

Fig. 1. The Cx30A88V protein forms gap junction plaques in the plasma membrane of the epidermis. Immunostainings of Cx26 in green (A and B) and Cx30 in red (C and D) in sole skin sections of wildtype and homozygous Cx30A88V mice show that both connexins are expressed in the stratum spinosum and stratum granulosum of the thick epidermis. Both wildtype connexins co-localize (yellow staining in the merged images (E and F)). The mutated Cx30A88V also co-localizes with Cx26 in the plasma membrane (detail view in (F)). Nuclei are in blue. Epidermal layers: St.B: stratum basale, St.S: stratum spinosum, StG: stratum granulosum, St.C: stratum corneum. Scale bars: 20 μm.

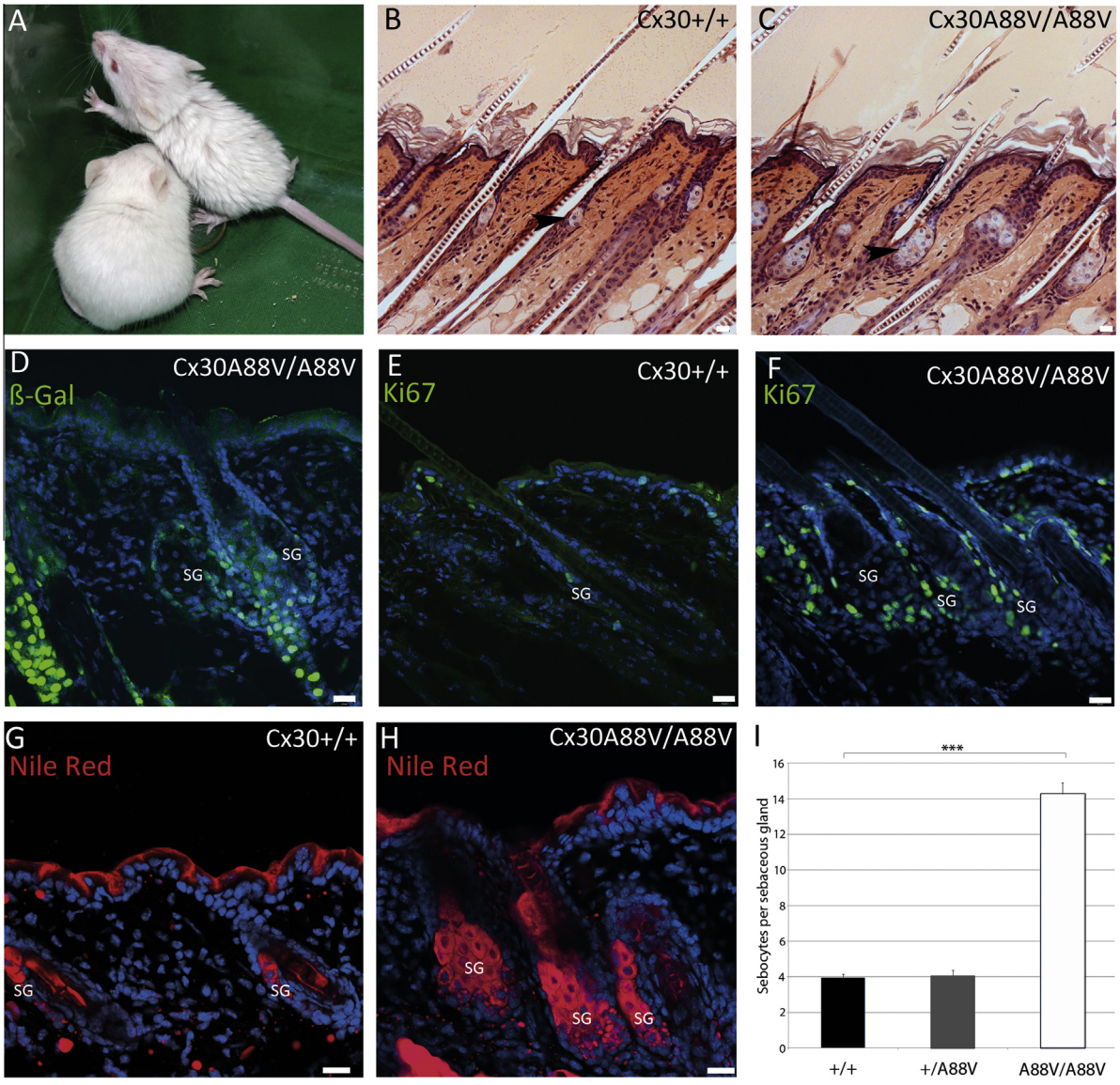

Fig. 2. Hyperproliferation of hair follicle associated sebaceous glands (SG). The fur of homozygous Cx30A88V mice (A, upper right mouse) appears greasy compared to wildtype littermates (A, lower left mouse). Hematoxylin and eosin (HE) staining of back skin reveals a strong enlargement of hair follicle associated sebaceous glands (SG) (B and C, arrows). ß-galactosidase reporter staining shows that Cx30A88V is expressed in transit amplifying cells of sebaceous glands (D). No cross-reaction of the ß-galactosidase antibodies with the wildtype control section was found (data not shown). The transit amplifying cells of the SG are hyperproliferative in homozygous Cx30A88V mice, as indicated by Ki67 immunofluorescence signals in green (E and F). Nile red staining detects lipid filled sebocytes (G and H). Counting of Nile red positive cells reveals that the SG of homozygous Cx30A88V mice includes 3.5 × more sebocytes than heterozygous or wildtype Cx30A88V littermates (I). Nuclei in blue. SG: sebaceous gland; Scale bars: 20 μm.

observed during the first hair cycle on postnatal day 10 and could be used to distinguish homozygous Cx30A88V mice from wildtype littermates, without exception. No age-dependent development of this phenotype could be observed. The oldest mice which were analysed were 12 months of age. No other abnormalities of the hair follicle organisation or number of hair follicles could be seen. Furthermore, no alterations in the frequencies of hair types were observed (Supplement Fig. 3). Heterozygous Cx30+/A88V did not show fur abnormalities. Sections and HE stainings of dorsal skin revealed a strong enlargement of hair follicle associated sebaceous glands (SG) in homozygous Cx30A88V mice (Fig. 2C) compared to wildtype littermates (Fig. 2B). Immunofluorescence analyses using antibodies against the NLS-ß-galactosidase reporter protein indicated that Cx30A88V is expressed in the transit amplifying cell layer of the sebaceous glands and in the outer root sheath of the hair follicle (Fig. 2D). Immunofluorescence staining of the proliferation marker Ki67 revealed that the transit amplifying cells of the sebaceous glands are hyperproliferative in homozygous Cx30A88V mice (Fig. 2E and F). To quantify the number of sebocytes per sebaceous gland, back skin sections were stained with the fluorescent lipid dye Nile red (Fig. 2G and H) and the number of sebocytes per sebaceous gland were counted. The number of sebocytes in homozygous Cx30A88V mice was elevated by a factor of 3.5 (Fig. 2I).

To investigate if other sebaceous glands are affected, which are not associated with hair follicles, cryosections of meibomian glands were perfomed. Meibomian glands are located at the inner rim of the eyelids and are not connected to hair follicles. ß-Gal and Ki67 stainings of meibomian gland sections revealed that Cx30A88V is also expressed at the proliferative layer of the glands,

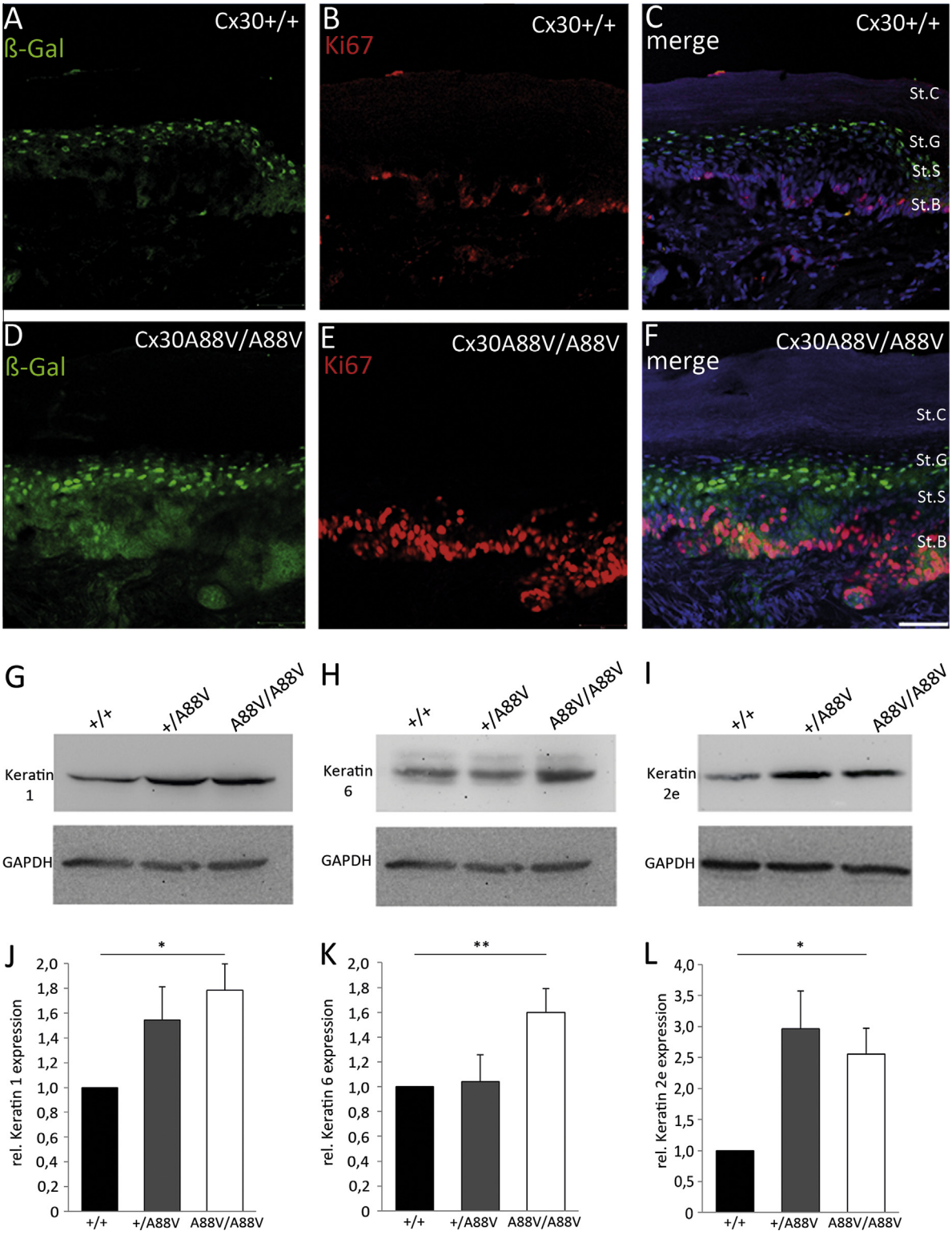

Fig. 3. Mild hyperkeratosis of palmoplantar epidermis of Cx30A88V mice. Immunostainings of ß-galactosidase reporter protein in green (A and D) and proliferation marker Ki67 in red (B and E) of wildtype and homozygous Cx30A88V mice. (G–L) Quantitative immunoblot analyses of keratin 1, keratin 6 and keratin 2e in paw epidermis (J–L) Statistical evaluation of protein amounts reveals a significant increase of keratin 1 (J), keratin 6 (K) and keratin 2e (L) protein level in homozygous Cx30A88V mice compared to wildtype. The keratin 1 and keratin 2e expression of heterozygous Cx30+/A88V mice seems to be elevated compared to wildtype, but does not reach statistical significance. $n = 4$ for Keratin 1 and Keratin 2e, $n = 6$ for Keratin 6.

but there is no obvious enlargment or hyperproliferation of meibomian glands in homozygous mice (Supplemental Fig. 4). This indicates that the hyperproliferation of sebaceous glands is restricted to those associated with the hair follicle.

3.4. Mild hyperkeratosis of palmoplantar epidermis

Morphological analyses with ß-Gal and Ki67 stainings of paw cryosections showed that some parts of the palmoplantar

epidermis of homozygous mice appeared to be slightly thickened and hyperproliferative compared to wildtype mice (Fig. 3A–F), but no obvious continuous hyperplasia of the epidermis could be observed by microscopy. However, quantitative keratin expression analyses of paw skin revealed a mild hyperkeratosis in homozygous Cx30A88V mice. Keratin 1 is expressed in all suprabasal layers (Supplemental Fig. 5A–C, in red) and used as a general marker for differentiation. Keratin 6 is weakly expressed during very late differentiation in the upper stratum granulosum (Supplemental Fig. 5A–C in green). Keratin 6 has been described to be upregulated in hyperproliferative skin [16]. Keratin K2e is expressed in the upper stratum spinosum and stratum granulosum (Supplemental Fig. 5D–F) and has been associated with hyperkeratotic epidermis [17]. Immunoblot analyses with antibodies against keratin 1, keratin 6 and keratin 2e revealed that the protein levels of all three keratins are significantly elevated in paw skin samples of homozygous Cx30A88V mice (Fig. 3J–L). In heterozygous mice there is also a tendency for higher keratin 1 and keratin 2e expression compared to wildtype mice, without reaching significance.

3.5. Impaired hearing function in Cx30A88V mice

Hearing thresholds were assessed by recordings of Auditory Brainstem Responses (ABR) evoked by tone burst and click stimuli. ABR wave forms in Cx30A88V mice had normal latencies and amplitudes (Fig. 4A). Compared to wildtype and heterozygous mice, homozygous Cx30A88V mice showed significantly elevated ABR thresholds in response to low-frequency tone bursts (Fig. 4B). In contrast, high-frequency ABR thresholds and DPOAE (Distortion Product Otoacoustic Emissions, Supplemental Fig. 6) amplitudes were improved in homozygous Cx30A88V mice compared to wildtype and heterozygous controls.

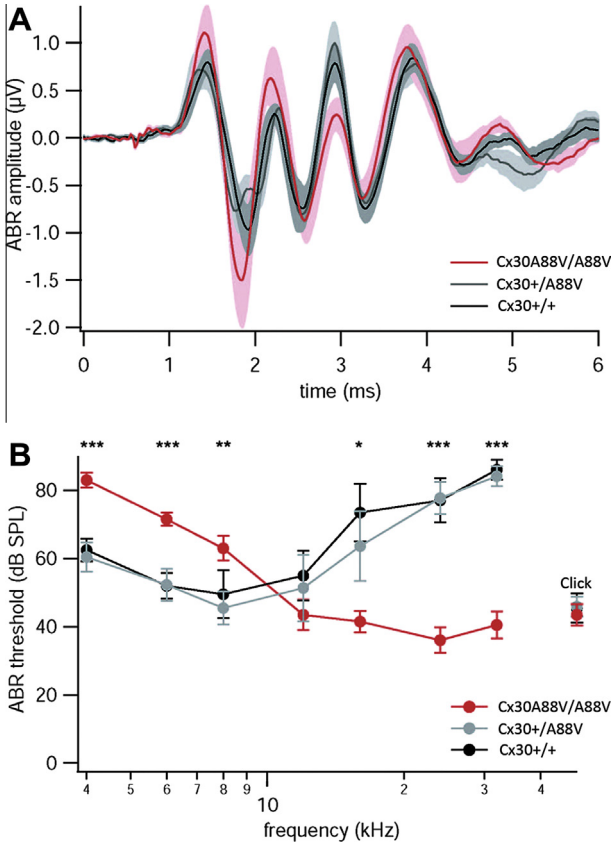

Fig. 4. Hearing function in Cx30A88V mice (A) Grand averages of ABR waveforms in response to 80 dB clicks from wildtype (black, $n = 9–10$), heterozygous (grey, $n = 8$) and homozygous (red, $n = 9$) Cx30A88V mice. (B) Homozygous Cx30A88V mice showed significant low-frequency hearing impairment as measured by ABR thresholds. In contrast, high-frequency thresholds were significantly lower than in wildtype and heterozygous control animals. Thresholds for 12 kHz tone bursts and clicks were unchanged.

4. Discussion

In this study we have generated and characterized a new transgenic mouse line expressing the human Clouston syndrome Cx30A88V mutation under the control of the endogenous Cx30 promoter.

Previously published cell culture experiments indicated a cytoplasmatic localization of the mutated Cx30A88V protein and revealed an improvement of trafficking when co-expressed with wildtype Cx30 [13,18]. A recent publication shows that the trafficking of Cx30A88V could not be rescued in transfected cells, neither by co-expressing wildtype Cx30 nor by co-transfection of wildtype Cx26. However, a part of the overexpressed Cx30A88V protein was always integrated into the plasma membrane [19]. Our immunofluorescence analyses of homozygous Cx30A88V mice confirm that the Cx30A88V protein can be integrated into plasma membranes of the keratinocytes (Fig. 1F). Apparently, under endogenous regulation the Cx30A88V protein can be incorporated in plaques of the plasma membranes without additional accumulation in the cytoplasm, which could be an effect of the overexpression system used in the cell culture studies [13,18,19].

Homozygous mice show an enlargement of hair follicle associated sebaceous glands, accompanied by hyperproliferation of the Cx30A88V expressing, Ki67 positive transit-amplifying cells of the sebaceous glands (Fig. 2). Additionally, homozygous Cx30A88V mice develop a mild palmoplantar hyperkeratosis, indicated by a higher expression of keratin 1, keratin 6, and keratin 2e proteins (3). Channel function analyses of a previous study revealed that channels composed of Cx30A88V show normal gap junctional coupling, but a gain-of-function 'leakage' of hemichannels, leading to an enhanced ATP-release into the extracellular medium [13]. Several studies indicate that this kind of hemichannel activity can promote proliferation [20,21]. It has been suggested that Cx45 hemichannels, which are expressed in the transit-amplifying cells of the subventricular zone of the brain, stimulate proliferation by releasing ATP. The authors propose a model involving the binding of released ATP to P2X receptors, which leads to Ca^{2+} release and modulation of cell cycle re-entry via ERK1/2 and cyclin D [22]. Of note, a recent publication showed that air-stimulated ATP release in keratinocytes can be blocked by hemichannel inhibitors and therefore indicates that connexin hemichannels are involved in ATP release from keratinocytes [23].

The molecular basis for the observed hyperproliferation in the Cx30A88V mouse line and the palmoplantar hyperkeratinization of Clouston syndrome patients may be similar to the mechanisms suggested for Cx45 hemichannels in the subventricular zone of the brain [22]. For example, an enhanced ATP release by 'leaky' Cx30A88V hemichannels could lead to the activation of Ca^{2+} dependent kinases via binding to Ca^{2+}channels. This could generally alter gene expression or lead to cell cycle re-entry respectively. Interestingly, the hyperproliferation of sebaceous glands observed in the Cx30A88V mice (Fig. 2) occurs only in hair follicle-associated glands. Free sebaceous glands like the meibomian glands do express Cx30A88V in a proliferative layer, but are not enlarged (Supplemental Fig. 4). This may indicate that the enhanced Cx30A88V hemichannel activity only leads to hyperproliferation of sebaceous glands, if the sebaceous glands are part of the pilo-sebaceous unit and therefore in an environment of Cx30A88V expressing outer root shaft cells of the hair follicle.

Since some heterozygous human patients with Clouston syndrome were reported to suffer from hearing loss [2,3], hearing analyes were performed with the Cx30A88V mouse line. Homozygous Cx30A88V mice showed impaired low-frequency hearing, consistent with endolymphatic hydrops due to changes in endolymph composition. The unusual finding of improved high-frequency thresholds in the mutants may easily remain undetected in a clinical setting. Enlarged DPOAE amplitudes confirm enhanced cochlear amplification in the high-frequency region. Future studies are required to understand whether this reflects a change in cochlear micromechanics or a protection from age-or noise-dependent hearing loss due to changes in cochlear homeostasis or in purinergic signaling due to altered gap junction permeability [24].

Taken together, the Cx30A88V mouse line represents some aspects of the human Clouston syndrome and can thus be used to further investigate the molecular mechanism of this disease, especially regarding hyperproliferation and hearing loss. However, clear phenotypic abnormalities occur only in homozygous Cx30A88V mice, although the Clouston syndrome is dominant in humans. Therefore, a high expression level of the Cx30A88V mutated protein seems to be required to cause a Clouston-like phenotype in mice. In autosomal dominant diseases the severity of the symptoms can be variable depending on varying expression ratios of wildtype to mutated protein [25]. Possibly, patients suffering from more severe symptoms express more mutated Cx30A88V than wildtype Cx30 protein. This hypothesis could explain the variability of symptoms and severity in Clouston syndrome. One study shows that patients who were originally diagnosed with Pachyonychia congenital carried the Clouston syndrome associated mutations Cx30G11R and Cx30A88V [26].

Clear differences between the Cx30A88V mouse line and Cx30A88V patients were observed regarding the fur phenotype, that is characterized by sebaceous hyperplasia but not by hair loss, a typical symptom of the human Clouston syndrome. These dissimilarities in mouse and human could be due to the fact that the fur of mice shows some general structural differences, e.g. the difference in hair types or the shorter duration of the growth phase compared to human hair. However, sebaceous gland hyperplasia could also be a new aspect of the human Clouston syndrome, which should be kept in mind in future clinical screenings.

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Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.040.

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